UNITED STATES PATENT APPLICATION

of

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for

USE OF ADENO-ASSOCIATED VIRUSES FOR DECREASING THE RADIOTHERAPY-INDUCED OR CHEMOTHERAPY-INDUCED RESISTANCE IN CANCER PATIENTS

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Use of Adeno-Associated Viruses for Decreasing the Radiotherapy-Induced or Chemotherapy-Induced Resistance in Cancer Patients

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The invention relates to the use of adeno-associated viruses for decreasing the radiotherapy-induced or chemotherapy-induced resistance in patients who suffer from a cancer which is to be treated by radiotherapy or chemotherapy.

Along with the removal by surgery malignant diseases have been treated by radiotherapy and/or chemotherapy thus far. However, the occurrence of side-effects, in particular the development of resistances, limits the use of cytostatic agents. Because of these side-effects chemotherapeutic agents can only be used to a limited extent. Thus, the dosage of a chemotherapeutic agent can only be a dosage which the patient tolerates. However, in most cases such a dosage only achieves a minor curative effect.

Therefore, it is the object of the present invention to reduce or attenuate the problem of resistance induced by radiotherapy or chemotherapy so as to improve the survival rate of cancer patients after a radiotherapy or chemotherapy. In addition, it shall be possible to reduce the subsequent doses of a radiotherapy or chemotherapy. The responsiveness of tumor cells to radiotherapy or chemotherapy shall also be improved.

This object is achieved by the subject matters defined in the claims.

According to the invention the development of a radiotherapy-induced or chemotherapy-induced resistance in patients suffering from a cancer to be treated by

radiotherapy or chemotherapy shall be reduced by using adeno-associated viruses.

It was found surprisingly that following a treatment with human non-pathogenic adeno-associated viruses (AAV) the tumor cells respond in a better way to a subsequent chemotherapeutic or radiotherapeutic measure.

The AAV virus is a human parvovirus which requires co-infection with a helper virus for a productive infection to take place. AAV infects humans in their infancy and is considered non-pathogenic, since no human disease could be correlated with the AAV infection (Adv. in Vir. Res. 1987, 32, 43-306).

According to the inventors' insight an AAV infection in combination with chemotherapeutic or radiotherapeutic measures increases the efficiency of a conventional therapy and reduces resistances which occur, so that a further therapy can be carried out in a more promising way than possible thus far. Any AAV viruses can be used according to the invention. The AAV-2 virus is used preferably.

In animal experiments carried out with immunodeficient naked mice into which small cell lung carcinoma cells were implanted subcutaneously, it could be shown that an AAV infection which was carried out at the same time as a chemotherapy resulted in a faster recession of the tumors. Relapses occurred after a short time in both groups. However, in the AAV-infected chemotherapeutic group they responded better to another chemotherapy than those of the group only treated by means of chemotherapy. This shows that an AAV infection can reduce or even avoid the development of resistances.

The use of the AAV viruses according to the invention can be made before, at the same time with or after the chemotherapy or radiotherapy. However, it is carried out preferably after

a first chemotherapeutic or radiotherapeutic treatment cycle.

In particular in the case of tumor kinds which *per se* show a poor response to a chemotherapy or radiotherapy it may be indicated to carry out the treatment before or together with the chemotherapy/radiotherapy to increase the efficiency of the therapy. By this the treatment doses can be lowered and therefore the side-effects can be reduced.

The radiotherapy or chemotherapy may be any radiotherapy or chemotherapy which is adapted to the cancer to be treated. Such therapies have been known for years and along with the removal of the tumor by surgery they represent the established method of curing cancer diseases or increasing the life expectancy of a patient by some time. Therefore, a person skilled in the art is perfectly familiar with the measures of a radiotherapy or chemotherapy.

The use of AAV viruses according to the invention can be applied to any cancer kinds, the best success being expectable in connection with colon cancers, pancreatic carcinomas and brain tumors (in particular glioblastomas). The small cell lung carcinoma (SCLC) can preferably be treated therewith.

The application according to the invention is made intravenously, by means of infusions, intratumorally, orally (also by means of inhalations) or cutaneously. In this connection, the virus is formulated in a suitable preparation adapted to the pathway of administration. For an intravenous (also as an infusion) and intratumoral administration it is preferred to provide the virus in a physiological common salt solution, Ringer's solution or PBS solution (phosphate-buffered salt solution), for a cutaneous administration it is preferred to provide it in the form of an ointment, suspension or gel, and for oral administration it is preferred to provide it in the form of an aerosol spray.

Depending on the patient's body weight the virus dose employed is 10^9-10^{10} AAV particles/kg body weight.

A pharmaceutical composition is also provided according to the invention which in addition to the chemotherapeutic agent (cytostatic agent) contains adeno-associated viruses, in particular AAV-2. All chemotherapeutic agents (cytostatic agents) common in tumor therapy thus far can be used separately or in combination as a chemotherapeutic agent, e.g. cisplatin, etoposide, methothrexate, doxorubicin, cyclophosphamide, trofosfamide, busulfane, cytarabin, fluorouracil, mercaptopurine, vinblastinesulfate, vincristinesulfate, bleomycinsulfate or mitomycin. Thus, it is preferred for an intravenous (also by means of infusion) and intratumoral administration to provide an injection solution, for a cutaneous administration to provide an ointment, for an oral administration to provide an aerosol spray. As a basis for the preparation of the infusion solution physiological common salt solution, Ringer's solution or PBS each are suitable in pure form or as a mixture. The amount of AAV depends on the patient's weight and is 10^9-10^{10} particles/kg body weight. In the pharmaceutical composition it is contained in an amount suitable for an average body weight of 70 kg. The accurate dosage of the pharmaceutical composition according to the invention is determined by a physician and depends on the patient's sex and weight, severity of the disease, kind of administration and planned duration of administration. A composition according to the invention may also contain conventional auxiliary agents. The common auxiliary agents such as carriers, binders, blasting agents, lubricants, solvents, solubilizers, release accelerators, release decelerators, emulsifiers, stabilizers, colorants of the taste correctives may be used as auxiliary agents.

The invention is explained in more detail by means of the attached figures.

Figure 1 shows a diagram of the protocol used for determining AAV-2-mediated drug-sensibilization. The proliferation of the SCLC cell lines after the infection and/or drug treatment was determined by the MTT assay (J. Immunol. Methods 1983, 56, pp. 55-63). The relative proliferation (A/Ao) was calculated by the ratio absorption of AAV-2-infected and/or drug-treated cells (A) to absorption of mock-infected untreated cells (Ao).

Figures 2a+b show the AAV-mediated sensibilization of the SCLC cell lines over cisplatin, the relative proliferation (A/Ao) of the SCLC cell lines, NCI-H209 (figure 2a) and NCI-H446 (figure 2b) following a mock infection (a: PBS alone; b: infected with a heat-inactivated gradient of Ad2-infected cells) or infection with various multiplicities of an infection with AAV-2 with (gray columns) or without (white columns) subsequent treatment with IC50 of cisplatin according to Table 1 (TCID, tissue culture infectious dose).

Figure 3 shows the AAV-mediated sensibilization of tumors in naked mice, derived from NCI-H209 cells (5 mice per group). The dose of cisplatin was 3 mg/kg body weight (weekly administration). The etoposide dose was 7.5 mg/kg body weight (administered three times a week). AAV-2 was administered weekly in a MOI of 10^8 TCID/animal. Arrows show the change of the treatment modalities: - interruption of the drug treatment and AAV-2 infection; + beginning of the treatment and the infection.

The invention is explained in more detail by way of the small cell lung carcinoma (SCLC). This carcinoma type is generally characterized by an initially effective chemotherapy and remission of the tumor. However, almost all of the patients suffer from a relapse which resulted from a resistance of the tumor cells to the first applied chemotherapy (usually cisplatin/etoposide). Therefore, it was tested in a model system with a human small cell lung carcinoma cell line whether an infection with AAV enhances the cytotoxic effect of the chemotherapeutic agents in the

cell culture and in tumors of immunodisturbed mice. It is shown that the AAV infection increases significantly the effectiveness of the chemotherapy of SCLC tumor cells and SCLC tumors.

Example 1

Sensibilization of SCLC cell lines over cisplatin and etoposide by AAV infection

Small cell lung carcinoma cell lines (NCI-H69, NCI-H164, NCI-H209 and NCI-H446) (Cancer Res., 1980, 40, 3502-3507; Cancer Res., 1985, 45, 2913-2923) were cultured in RPMI-1640 medium (Eurobio, Raunheim, Germany). HeLa cells were cultured in DMEN (Eurobio, Raunheim, Germany) All growth media were supplemented with glutamine (Eurobio, 1 %), antibiotics (penicillin and streptomycin) and 10 % heat-inactivated fetal calf serum (PAA; Liz, Austria). The cultures were incubated at 37°C in a damp atmosphere with 5 % CO_2 and tested for mycoplasma contaminations at regular intervals.

The adeno-associated virus type 2 (AAV-2) was replicated in HeLa cells using adenovirus type 2 (Ad-2) as a helper. AAV was purified in a cesium-chloride gradient and titrated as described in J. Gen. Virol., 1994, 74, 2655-2662. The adenovirus type 2 inocula were clarified supernatants of Ad2-infected HeLa cells.

The SCLC cells were suspended in PBS and incubated with purified AAV-2 in the indicated multiplicities of infection (MOI). After 45 min at 37°C, unbound absorbed virus was removed by washing using PBS and the growth medium was supplemented. Either PBS or the heat-inactivated fraction (56°C, 30 min) of a CsCl gradient of Ad-2-infected cells alone was used for the controls (mock infection), the density (1.14 g/cm^3) of the AAV-2-containing fraction which was used for the AAV-2 purification being indicated in the

respective experiments. The volume/cell ratio of these experiments was 50 times greater (5 ml/16^6 cells) than the one used for the AAV infections.

The AAV-2-infected or mock-infected cells were treated with cisplatin (Astra Medica, Frankfurt/Main, Germany) or etoposide (Bristol, Munich, Germany) or both in a ratio [cisplatin : etoposide = 1:2.5], the pharmacons, dissolved in PBS, being added to the medium in the indicated concentration.

The proliferation of the SCLC cells after the infection with AAV-2 and/or treatment with chemotherapeutic agents was determined by the modified MTT test (J. Immunol. Methods, 1983, 56, 55-63). After the infection or mock infection, the cells were placed in plates having 24 wells with a density of 10^5 cells/well and treated with the chemotherapeutic agents in the indicated concentrations. After six days (NCI-H69, NCI-H446) or eight days (NCI-H146, NCI-H209), MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide; Sigma, Deisenhofen, Germany) was added to the culture up to a final concentration of 0.5 mg/ml. The cultures were then incubated at 37°C for 4 h to permit a reduction of MTT into blue formazan by mitochondrial dehydrogenases (Arch. Biochem. Biophys., 1993, 303, 474-482), which indicates active proliferation of the cells. The cells were centrifuged, washed with PBS, and formazan was solubilized in isopropanol. The precipitated proteins were pelleted by centrifugation (1000 rpm, 15 min) and 200 µl samples of the supernatant were measured to determine the optical density at 540 nm (OD540), OD690 being used as a reference and a Titertek Multiskan plus MKII densitometer being employed (Lab Systems, Finland).

The relative proliferation (A/Ao) was defined as the ratio of the absorption (A) measured in the supernatant of AAV-2-infected and/or pharmacon-treated cells, compared with the absorption measured for the supernatant of the mock-infected cells and untreated control cells (Ao). The IC50 value was

defined as pharmacon concentrations resulting in a 50 % inhibition of proliferation.

The AAV-2-infected cells (MOI values as indicated) or mock-infected cells were placed in plates having 24 wells and treated with cisplatin. After six or eight days, the relative proliferation was determined. In addition, kinetic studies were carried out to determine the optimum treatment modalities for SCLC cells after an AAV infection. It was shown in these investigations that the AAV-mediated sensbilization reached a maximum after one to three hours following the infection. In this investigation the chemotherapeutic agents were administered three hours after the AAV infection. In order to exclude effects which are due to factors still present after the purification with the CsCl gradient and the Ad2 heat inactivation, a control of the mock infection with PBS was carried out in addition to the mock infection with the fraction of the respective gradient of a cell lysate of Ad2-infected cells.

The relative proliferation of NCI-H209-SCLC cells and NCI-H446-SCLC cells was measured after the infection with various multiples of the infectious units (MOI) of AAV-2 (10^1-10^5 tissue culture infectious dose (TCID) per cell) with and without subsequent treatment with cisplatin with the IC50 values listed in Table 1.

Table 1

Concentration of the chemotherapeutic agents which result in a 50 % inhibition of the proliferation (IC50) of the SCLC cell lines

NCI-H69	0.2	0.26	0.08/0.2
NCI-H146	0.11	0.025	0.008/0.02
NCI-H209	0.007	0.053	0.006/0.015
NCI-H446	0.15	0.21	0.042/0.105

As shown in Table 1, the SCLC cell lines NCI-H69 and NCI-H446 showed a high intrinsic resistance towards both pharmaceutical preparations, the susceptibility to cisplatin/etoposide treatment being lesser, whereas NCI-H146 cells were highly susceptible to etoposide and the NCI-H209 cells were highly susceptible to both pharmacons.

As follows from figure 2 (a+b), the AAV-2 infection resulted in a decrease of the proliferation rate of the cisplatin-treated cells with a MOI of 10^3 to 10^4 TCID/cell. The infection with a MOI of 10^5 TCID/cell resulted in no further increase. No significant inhibition of the proliferation was observed after the infection with lower MOI values of AAV-2 or after the mock infection, which indicates a specific effect due to the infection with high MOI of AAV-2. The relative proliferation of AAV-2-infected (10^{3-5} AAV/cell) and with cisplatin-treated (IC50) cells was lowered to 0.29 in NCI-H446 cells and to 0.25 in NCI-H209 cells as compared to the relative proliferation in (IC50) cells treated only with cisplatin (0.59 in NCI-H446 cells and 0.5 in NCI-H209 cells).

Example 2

Quantification of the AAV-2-mediated pharmacon sensibilization of SCLC cell lines

In order to quantify the sensibilization of cells over chemotherapeutic agents after infection with AAV-2, dose-response curves were prepared. The relative proliferation of the cell lines was determined after a mock infection (PBS) or AAV infection with 10^3 or 10^4 TCID/cell and subsequent treatment with various concentrations of cisplatin or etoposide or a combination of both pharmaceutical preparations (Table 2).

Table 2

Sensibilization factor of SCLC cell lines which were treated with cisplatin and/or etoposide and were infected with AAV.

Cell line	Chemotherapeutic agent	Sensibilization factor (A/Ao)* 10^4 Ip. AAV/Cell
NCI-H69	Cisplatin	1.43
	Etoposide	1.30
	Cisplatin/etoposide	1.45
NCI-H146	Cisplatin	1.37
	Etoposide	1,38
	Cisplatin/etoposide	1.33
NCI-H209	Cisplatin	2.33
	Etoposide	2.12
	Cisplatin/etoposide	2.00
NCI-H446	Cisplatin	2.50
	Etoposide	3.00
	Cisplatin/etoposide	3.00

* The relative proliferation (A/Ao) was calculated by the ratio absorption of AAV-2-infected and/or pharmacon-treated (A) cells to absorption of the mock-infected, untreated controls (Ao)

The sensibilization factors (SF) were defined as the ratio of the IC50 values of infected cells compared with the IC50 values of mock-infected cells. The sensibilization factor

indicates the factor by which the concentration of a chemotherapeutic agent can be reduced after an infection with AAV-2 to obtain the same degree of proliferation inhibition. As summarized in Table 2 the sensibilization by AAV-2 in NCI-H69 and NCI-H146 was moderate (maximum SF about 1.4 with a MOI of 10^4 TCID/cells). The infection of NCI-H209 or NCI-H446 induced a more significant MOI-dependent sensibilization (maximum SF about 3 (NCI-H446) and 2.3 (NCI-H209) with a MOI of 10^4 TCID/cell). The AAV-2-mediated sensibilization did not depend on the chemotherapeutic agent employed.

Example 3

AAV2-mediated pharmacon sensibilization of NCI-H209-derived tumors in naked mice

H209 is a cell line which is derived from a tumor which was not treated chemotherapeutically before cultured and is not resistant to drugs.

Female naked mice (CD1-nu/nu) from Iffa Credo (Brussels, Belgium) were kept in isolators and were given water and food as desired. Experimentally growing SCLC cells (H209) were injected subcutaneously into the side of the six-week-old mice (10^7 cells in 100 μl PBS per animal). Five months after the inoculation of the cells, when the tumors had reached an average volume of 200 mm^3, the animals were infected weekly with AAV-2 (intratumoral injection of 10^8 tissue culture infectious doses (TCID)) and/or with chemotherapeutic agents by intraperitoneal injection of 3 mg/kg body weight cisplatin (weekly) and 7.5 mg/kg body weight etoposide (three times a week). Details of the beginning and end of the treatment are indicated in figure 3. In each group (control, chemotherapeutic treatment, AAV2 infection, treatment + infection) five animals were received. The infected and non-infected animals were kept in separate isolators. The tumor diameters were measured weekly

and the tumor volume was determined by the formula tumor volume = ½ x width x depth x height. The relative tumor volume (V/Vo) was determined for each animal and each time (ratio of the tumor volume [V] compared with the tumor at the beginning of the treatment ([Vo]).

As follows from figure 3, the treatment with chemotherapeutic agents resulted in a rapid decrease of the tumor volumes and a complete regression after three weeks of treatment. The combination of chemotherapy with AAV2 infection resulted in a more rapid decrease of the tumor volumes compared with animals which had only received a chemotherapy, which indicates a sensibilization of the pharmacon-treated tumor cells by AAV-2. The infection with AAV-2 alone had no significant effect, and the tumor volumes increased to the same extent as did the untreated controls. The treatment was discontinued after complete regression of the tumors and was resumed in the case of a relapse. The treatment of relapses was less effective with animals, which had only received drug treatment, as compared to the animals infected with AAV and treated chemotherapeutically. This shows the development of a resistance to the initial treatment at least in the chemotherapeutically treated animal group. The relapses in AAV-2-treated animals were still susceptible to cisplatin and etoposide treatment but the tumor regression was slower as compared to the regression of the initial tumors. In 3 of 5 AAV-2-infected animals the tumors regressed completely in week 9, in contrast to the tumors of animals which were only treated with chemotherapeutic agents, a complete regression of the tumor not being induced.